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Original Article

Long noncoding RNA HOXA-AS3 promotes pancreatic cancer progression by sponging miR-29c to upregulate CDK6

Jianping Song[1*], Wei Feng[2*]

[1]*The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang, P. R. China;* [2]*Puyan District Community Health Center, Hangzhou, Zhejiang, P. R. China.* [*]*Equal contributors and co-first authors.*

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Abstract: Background: Long noncoding RNAs (lncRNAs) HOXA-AS3 have been reported as potential oncogenes. Nevertheless, the molecular mechanism of HOXA-AS3 in pancreatic cancer (PC) progression remains unknown. Material/Methods: Quantitative real-time (qRT) PCR assay was performed to detect the expression levels of HOXA-AS3, miR-29c in PC specimens. Then, si-HOXA-AS3, miR-29c mimics, miR-29c inhibitors, or vector-CDK6 plasmids were transfected into PC cell lines to change the expression levels of HOXA-AS3, miR-29c, or CDK6. Luciferase reporter assays were performed to identify correlations between miR-29c, HOXA-AS3, and 3'UTR of CDK6. The ability of cell proliferation was assessed by cell counting and subcutaneous tumor growth assays. Results: HOXA-AS3 level was upregulated in PC, and its knockdown suppressed PC cells proliferation, whereas miR-29c antagonized the regulatory effect of HOXA-AS3 knockdown by directly binding to HOXA-AS3. Moreover, CDK6 was a potential target of miR-29c and miR-29c exerted anti-proliferation effects through CDK6. Conclusions: HOXA-AS3 accelerated growth of PC cells partially by regulating the miR-29c/CDK6 axis, which could be used as a potential therapeutic target in PC treatment.

Keywords: lncRNA HOXA-AS3, pancreatic cancer, miR-29c, CDK6

Introduction

Pancreatic cancer (PC), one of the most lethal malignant tumors, causing 432,242 new deaths in 2018 [1]. Treatment advances in recent decades did not improve the poor prognosis of PC and the 5-year survival rate of patients with PC was still less than 8% [2]. The progress of treatments for PC has been retarded due to the high invasiveness and strong resistance to chemotherapy [3]. Therefore, it is important to identify novel mechanisms in pathogenesis of PC. Traditionally, the researchers mainly focused on the genes that encoded proteins. However, these genes only accounted for a small percentage (1-2%) of the whole human genome and the majority of the genome encoded large numbers of regulatory noncoding RNAs [4].

LncRNAs are defined as transcripts without protein-coding potential [4]. LncRNAs participated in large range of cellular processes, including proliferation, differentiation and pluripotency maintenance though chromatin remodeling and epigenetic modification [4]. Furthermore, many studies have shown that several lncRNAs were involved in pancreatic cancer development and progression [5]. Previous chips results reported that the expression of lncRNA HOXA-AS3 was elevated in PC tissues compared to the matched normal tissues [6]. Upregulation of lncRNA HOXA-AS3 could promote the proliferations of many tumors, such as lung cancer and glioma [7, 8]. Mechanistically, lncRNA HOXA-AS3 could sponge miRNAs to inhibit mRNA degrading or interact with proteins directly [7, 9]. However, the relationship between lncRNA HOXA-AS3 and PC and its underlying mechanisms were still unclear.

In this study, expression changes of HOXA-AS3 were verified in PC tissues, while elevated expression of HOXA-AS3 might be associated with a worse prognosis. Next, downregulation of HOXA-AS3 in PC cell lines impaired the proliferations. Finally, HOXA-AS3 was found to poten-

tially regulate the miR-29c/CDK6 axis by sponging miR-29c. In sum, these studies reveal molecular mechanisms of how lncRNA HOXA-AS3/miR-29c/CDK6 axis controls PC proliferation.

Methods and materials

Specimens collection

This study was approved by the Second Affiliated Hospital of the School of Medicine of Zhejiang University Review Board and the ethics committees of Zhejiang University. Tissues from 63 patients with PC were collected from January 2013 to January 2018 and the informed consents were obtained.

Reagents and plasmids

The sequences of siRNA were 5'-UCUAUUCUCGCAAGGGAAATT-3' (Si/HOXA-AS3-1) and 5'-UUCUCCGAACGUGUCACGUTT-3' (siNC) [9]. The sequences of HOXA-AS3 and 3'UTR of CDK6 were cloned from genomic DNA and inserted to a luciferase reporter plasmid. Site mutagenesis technology was used to construct the mutant plasmids. A primary antibody against CDK6 (Cell Signaling Technology) and anti-β-actin (Cell Signaling Technology) and a secondary antibody (anti-rabbit IgG, Santa Cruz Biotechnology) were purchased.

Cell culture

Pancreatic cancer cell lines (Panc-1, Aspc-1, sw1990, and Bxpc-3) and immortal normal pancreas ductal epithelial cell (HPDE) were purchased from the cell bank of the Chinese Academy of Science, Shanghai. Cells were maintained in DMEM or RPMI 1640 (Gibco) supplemented with 10% FBS (Gibco), 2 mmol/l L-glutamine (Hyclone), and penicillin (50 U/ml)/streptomycin (50 μg/ml) (Hyclone) at 37°C under 5% CO_2 in a humidified chamber.

qRT-PCR

Total RNA was isolated from cells and then reversely transcribed to synthesize cDNA template. Next, qRT-PCR was performed by using cDNA template and specific PCR primers. The PCR primers were as follows:

HOXA-AS3: Forward, 5'-TTCATCCGCTGCTGCATCCAAGG-3'; Reverse, 5'-GCAAAGCACTCCATGACGAA-3'. Actin: Forward, 5'-CTCCATCCTGGCCTCGCTGT-3'; Reverse, 5'-GCTGTCACCTTCACCGTTCC-3'. qRT-PCR was performed on the 7500 Fast Realtime PCR system (Applied Biosystem) using SYBR Green agent. All PCR assays were repeated for three times.

Western blot

Protein (50 ug/sample) from cell lysates was fractionated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes. After blocking, membranes were incubated for 2 hours at room temperature with primary antibodies against CDK6 (Cell Signaling Technology) and anti-β-actin (Cell Signaling Technology) [10].

Cell growth counting and clone formation assays

For cell growth counting assays, Panc-1 and Bxpc-3 were plated in 96-well plates at a density of 1.5×10^3 cells per well. The cells were tested followed by cell counting kit-8 at 450 nm every day. For clone formation assays, pancreatic cancer cells were seeded into 6-well plates at a density of 5×10^3 cells per well. After 2 weeks of growth, the cells were fixed in methanol and dyed with methylrosanilnium chloride solution at a 0.01% concentration for counting [10].

Luciferase reporter assay

Synthesized luciferase reporter plasmids, miR-29c mimics and inhibitors, were transiently transfected into the Panc-1 and Bxpc-3 cells. The whole cell lysate was collected 24 hours after transfection and the luciferase activities were measured by the luciferase reporter assay kit (Invitrogen).

Subcutaneous tumor growth

All animal experiment procedures were approved by the Medicine Committee for the Use and Care of Animals of Zhejiang University. A total of 2×10^6 Panc-1 cells were injected to the left scapular region of nude mice (male, 6-week old, purchased from Slaccas Laboratory Animal) and tumor volumes were monitored every 3 days.

Statistical analysis

Data are shown as the mean ± one standard deviation (SD) and analyzed by a *t*- test. A Chi-

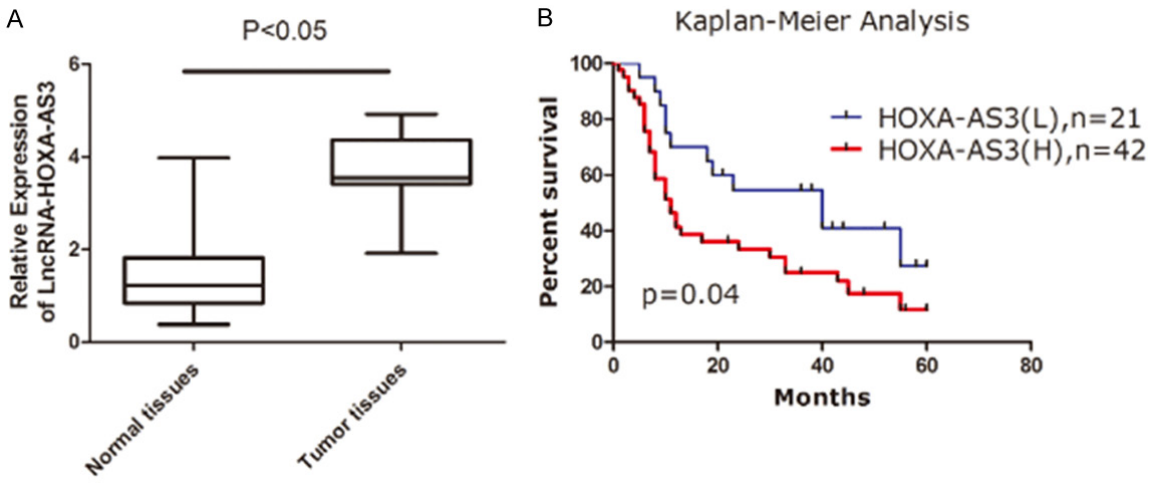

Figure 1. qRT-PCR results of lncRNA HOXA-AS3 in PC specimens (*: P<0.05). A. qRT-PCR to detect NPM1 in paired human PC specimens (tumor vs. peri-tumor). B. Survival analysis of PC patients by Kaplan-Meier plots and log-rank tests. Patients were categorized by high and low expression of lncRNA HOXA-AS3 based on qRT-PCR results. H, high; L, low.

Table 1. Correlation analysis of the clinicopathological parameters with the level of lncRNA-HOXA-AS3 expression in 63 patients with PC (*P* value: Chi-squared test; N.S: P>0.05)

Variables	N	LncRNA-HOXA-AS3		P
		High (n=42)	Low (N=21)	
Gender				N.S
Male	41	27	14	
Female	22	15	7	
Age				N.S
<60	31	22	9	
>60	32	20	12	
Differentiation				N.S
Well	25	13	12	
Poorly	38	29	9	
Lymph node metastasis				0.03
Yes	34	27	7	
No	29	15	14	
Classification of TNM				0.03
I-II	26	13	13	
III-IV	37	29	8	

squared test was used to analyze the correlation between clinicopathological parameters and the level of lncRNA-HOXA-AS3 expression. The Kaplan-Meier analysis and log-rank test were used to calculate the postoperative survival time. A *P*<0.05 was considered statistically significant. All the processes of statistical analysis were performed using IBM SPSS Statistics Version.20 software.

Results

LncRNA-HOXA-AS3 was upregulated in PC and predicted a poor prognosis

A total of 63 specimens from PC patients were collected and the total RNAs of tumor and the adjacent normal tissues were extracted for further investigations. Expression of HOXA-AS3 was elevated in PC tissues compared to the matched adjacent normal tissues (P<0.05) (**Figure 1A**). The correlation between expression of HOXA-AS3 and several clinicopathological parameters was calculated by Fisher's exact test and the results show that a higher expression of HOXA-AS3 correlated with the higher TNM stage (P=0.03) and positive lymph node metastasis (P=0.03) (**Table 1**). Combined with postoperative fellow-up data, patients with relatively low HOXA-AS3 expression were found to have a better prognosis compared to those with high HOXA-AS3 expression (P<0.05) (**Figure 1B**).

LncRNA-HOXA-AS3 could promote the proliferation ability in PC cell lines

Higher expression level of HOXA-AS3 in PC tissues was also found to predict worse prognosis. Therefore, HOXA-AS3 might promote the growth of PC cells. To test this hypothesis, the expression levels of HOXA-AS3 in five cell lines (four pancreatic cancer cell lines (Panc-1, Bxpc-3, Aspc-1, Sw1990) and one normal pancreatic ductal cell (HPDE)) (**Figure 2A**) were determined

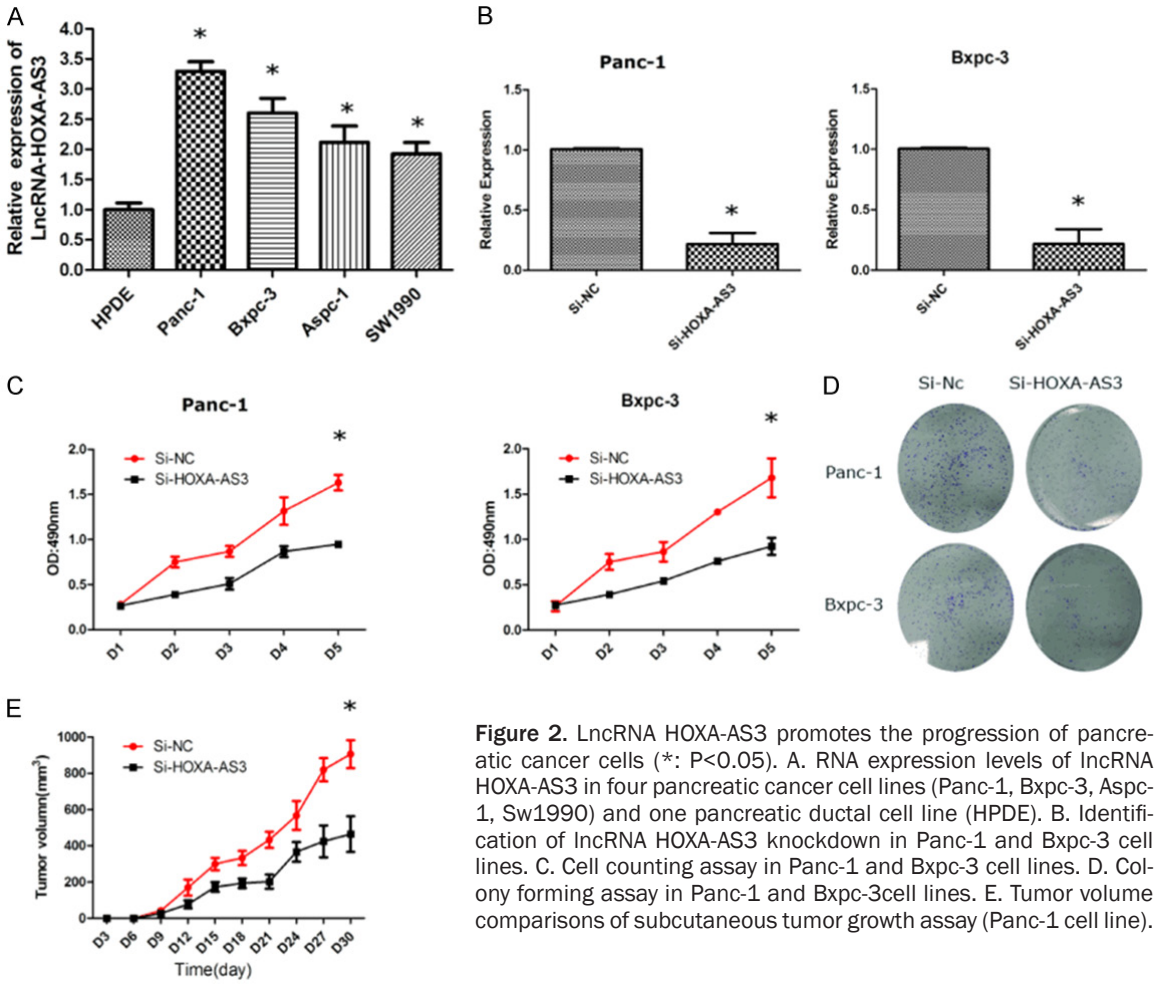

Figure 2. LncRNA HOXA-AS3 promotes the progression of pancreatic cancer cells (*: P<0.05). A. RNA expression levels of lncRNA HOXA-AS3 in four pancreatic cancer cell lines (Panc-1, Bxpc-3, Aspc-1, Sw1990) and one pancreatic ductal cell line (HPDE). B. Identification of lncRNA HOXA-AS3 knockdown in Panc-1 and Bxpc-3 cell lines. C. Cell counting assay in Panc-1 and Bxpc-3 cell lines. D. Colony forming assay in Panc-1 and Bxpc-3cell lines. E. Tumor volume comparisons of subcutaneous tumor growth assay (Panc-1 cell line).

and and HOXA-AS3 were relatively higher in the pancreatic cancer cell lines. Next, expression of HOXA-AS3 in Panc-1 and Bxpc-3 cell lines was downregulated and cell growth and colony forming abilities were dramatically impaired (**Figure 2B-D**) in these two cell lines. Panc-1 cells were also injected into subcutaneous region of nude mice and this validated that downregulation of HOXA-AS3 in Panc-1 cells slowed the tumor growth speed *in vivo* (**Figure 2E**).

LncRNA-HOXA-AS3 could sponge miR-29c in PC cell lines

Previous study reported lncRNA HOXA-AS3 could interact with miR-29c by special complementary sequences [9]. Expression of miR29c was elevated in lncRNA HOXA-AS3 knockdown cell lines. Furthermore, lncRNA HOXA-AS3 luciferase reporter plasmid (wild type) was constructed with mutated complementary sequences (mutant type) to preventing its binding with miR-29c (**Figure 3A**). The results show that miR-29c mimics could inhibit the luciferase intensities in wild type. The miR-29c mimics were also transfected with mutant type and the repression was no longer obvious (**Figure 3B**). Opposite results were obtained by transfecting miR-29c inhibitor (**Figure 3C**). Moreover, knockdown of lncRNA HOXA-AS3 could elevate the expressions of miR-29c in these two cell lines (**Figure 3D**). Expression of miR-29c in the 63 specimens was also determined and the results showed that miR-29c was down-regulated in tumor tissues (**Figure 3E**). A mild correlation was shown between lncRNA HOXA-AS3 and miR-29c in tumor tissues (P<0.05) (**Figure 3F**).

LncRNA-HOXA-AS3 regulated the miR-29c/CDK6 axis

Next, miR-29c was shown to inhibit expression of CDK6 by interacting with the 3'-UTR of CDK6

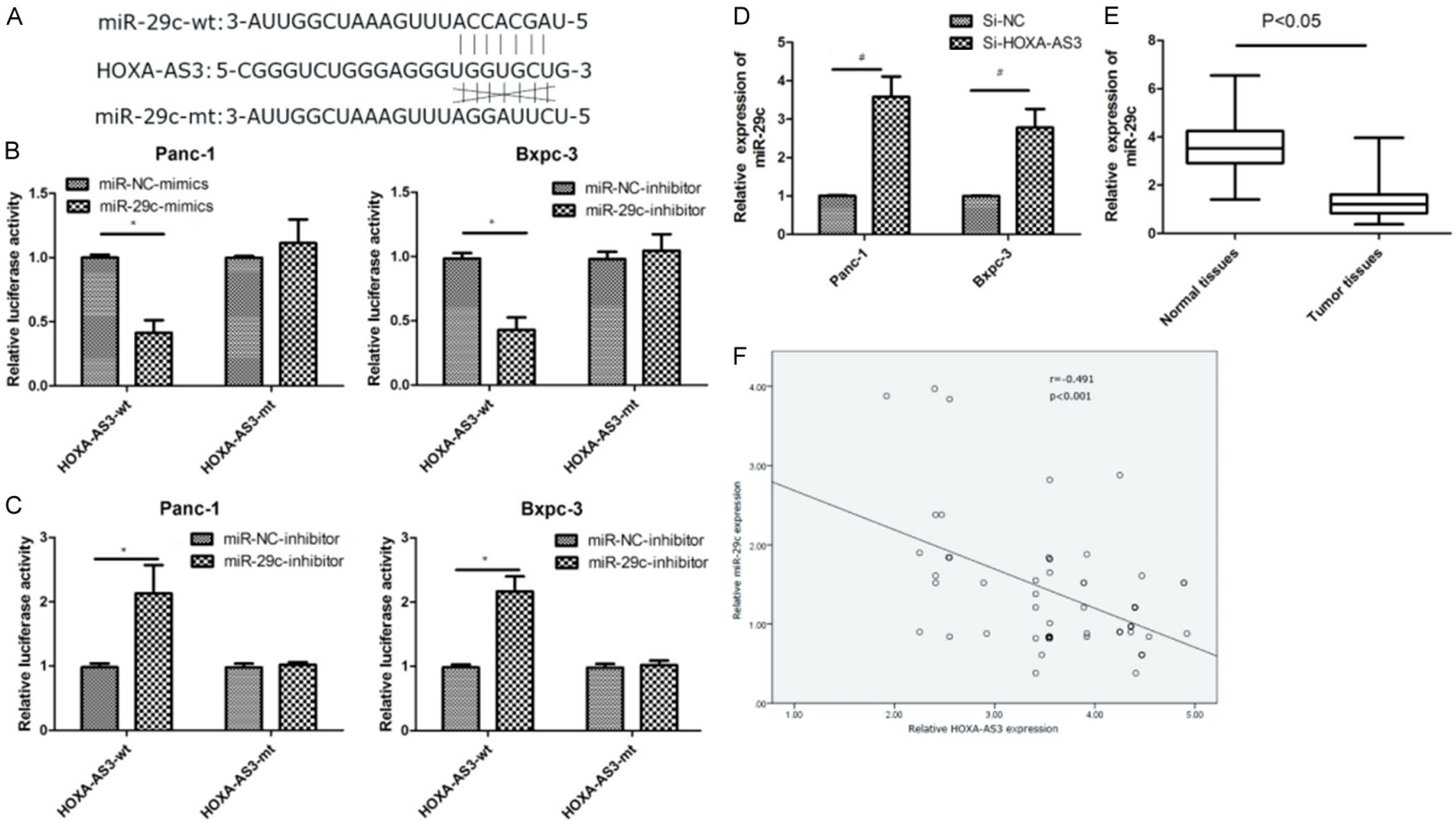

Figure 3. LncRNA HOXA-AS3 sponges miR-29c in pancreatic cancer cells (*: P<0.05). A. Putative binding site of miR-29c on the HOXA-AS3 and the mutation in the predicted seed region. B. Luciferase reporter assay for lncRNA HOXA-AS3 in Panc-1 and Bxpc-3 cell lines (miR-29c mimics). C. Luciferase reporter assay for lncRNA HOXA-AS3 in Panc-1 and Bxpc-3 cell lines (miR-29c inhibitors). D. The miR-29c expressions in lncRNA HOXA-AS3 knockdown cell lines. E. Relative miR-29c expressions in PC and matched adjacent tissues. F. The correlation analysis between lncRNA HOXA-AS3 and miR-29c expressions in PC tissues.

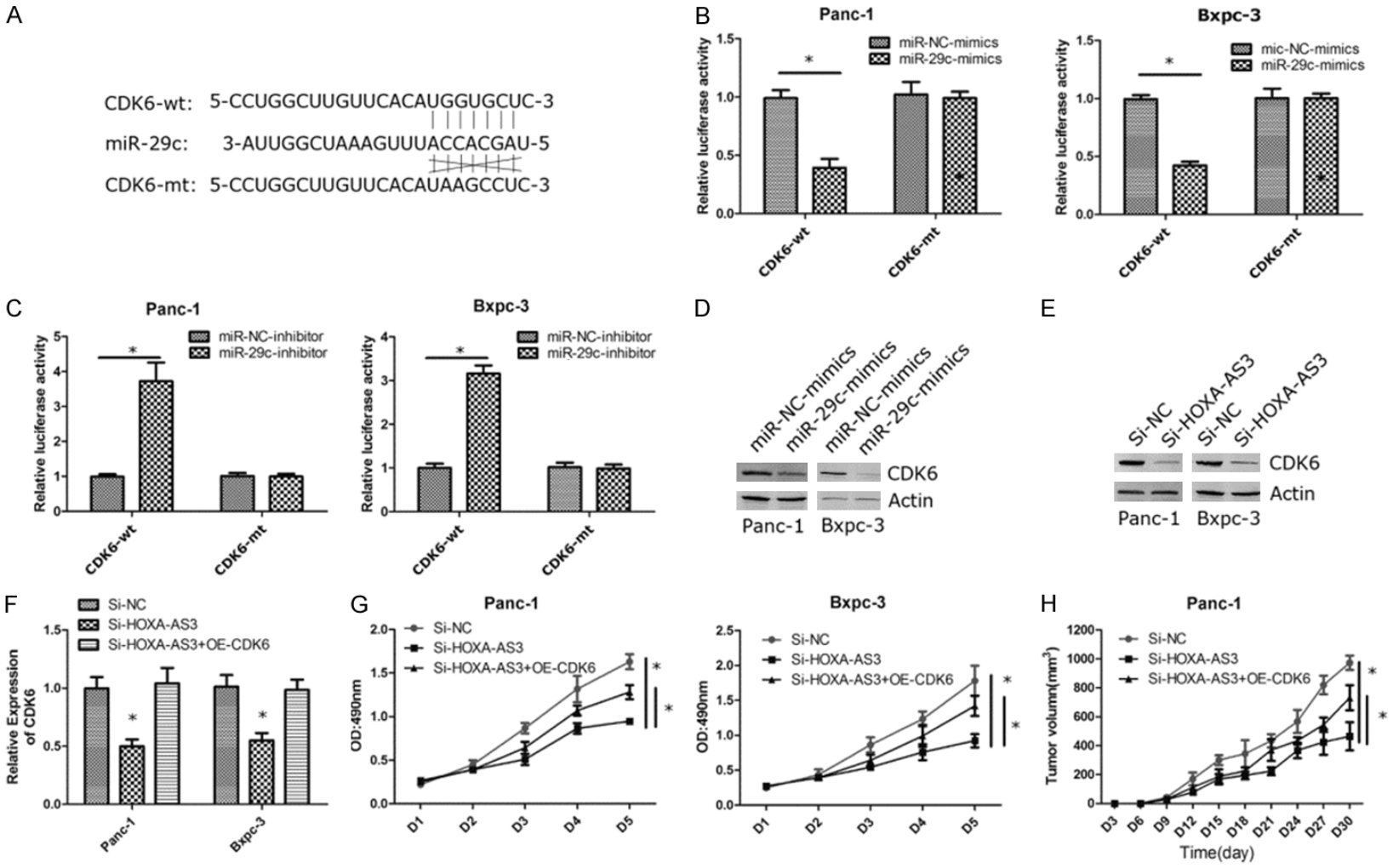

Figure 4. LncRNA HOXA-AS3 regulates the proliferation via miR-29c/CDK6 axis. A. Putative binding site of miR-29c on the CDK6 and the mutation in the predicted seed region. B. Luciferase reporter assay for 3'-UTR of CDK6 in Panc-1 and Bxpc-3 cell lines (miR-29c mimics). C. Luciferase reporter assay for 3'-UTR of CDK6 in Panc-1 and Bxpc-3 cell lines (miR-29c inhibitors). D. Protein expression changes of CDK6 in Panc-1 and Bxpc-3 cell lines (miR-29c mimics). E. Protein expression changes of CDK6 in Panc-1 and Bxpc-3 cell lines (Si-lncRNA HOXA-AS3). F. Restore CDK6 expression in Panc-1 and Bxpc-3 cell lines (Si-lncRNA HOXA-AS3). G. Cell counting assay in Panc-1 and Bxpc-3 cell lines (Si-lncRNA HOXA-AS3+CDK6 overexpression). H. Tumor volume comparisons of subcutaneous tumor growth assay (Panc-1cell line) (Si-lncRNA HOXA-AS3+CDK6 overexpression).

(**Figure 4A**). Furthermore, wild type and mutant type plasmids of 3'-UTR of CDK6 were constructed and co-transfected with miR-29c mimics and miR-29c inhibitor, respectively. The results showed that miR-29c could regulate luciferase intensities in wild type of 3'-UTR of CDK6 (**Figure 4B** and **4C**). Furthermore, miR-29c mimics or lncRNA HOXA-AS3 knockdown could repress the expression of CDK6 (**Figure 4D** and **4E**). Finally, expression of CDK6 in lncRNA HOXA-AS3 knockdown cells was restored and CDK6 could partially reverse the effects of lncRNA HOXA-AS3 on controlling the pancreatic cell proliferation (**Figure 4F-H**).

Discussion

The competing endogenous RNA (ceRNA) theory hypothesizes that a series of lncRNAs can function as molecular sponges of miRNAs to regulate target mRNAs expression and are involved in many tumorigenic and developmental processes [11]. In this study, several pieces of evidence supporting a critical role for HOXA-AS3 in tumor-promoting effects by targeting miR-29c/CDK6 axis. First, expression of HOXA-AS3 was elevated in PC specimens and a higher expression of HOXA-AS3 indicated a worse prognosis. Second, HOXA-AS3 could interact with miR-29c via special sequence and this sequence was also the binding site between miR-29c and CDK6, suggesting that HOXA-AS3 might regulate the expression of CDK6 by sponging miR-29c. Third, restoring CDK6 could partially reverse the effects of HOXA AS3 knockdown, which indicated that CDK6 might be the potential target of HOXA-AS3.

Emergent evidence indicates that miR-29c plays a role in PC cell growth, invasion, and migration by targeting ITGB1 [12]. miR-29c could also promote the chemosensitivity of PC cells by targeting USP22-mediated autophagy [13]. Moreover, the relationship between lncRNAs and miR-29c was also analyzed and lncRNA TUG1 could affect cell proliferation, invasion and migration via sponging miR-29c in PC [14]. CDK6 was a cyclin-dependent serine-threonine kinase and played a key role in reacting to mitogenic or pro-proliferative signals [15]. CDK6 induced cell-cycle arrest and senescence in many tumors and could be used as a potential therapeutic target in molecular treatment [16, 17]. miR-29c could inhibit the expression of CDK6 in bladder cancer [18]. Consistent with these reports, we verified that miR-29c was downregulated in PC tissues and could regulate the expression of CDK6 in PC cell lines.

Our study also has several limitations. First, the effects of HOXA-AS3/miR-29c axis and miR-29c/CDK6 axis had been validated in previous study and this study just connected these three molecules to build a new axis. However, no new molecules was identified from this study. Second, the relationship between HOXA-AS3 and tumor invasion and migration had not been studied in our study. Finally, miR-29c/CDK6 axis was one of the downstream pathways of HOXA-AS3 and more downstream pathways needed to be found in the future.

In conclusion, HOXA-AS3 could promote the proliferation of PC cells partially by regulating miR-29c/CDK6 axis and this new molecule could be used as a potential therapeutic target for PC treatment.

Disclosure of conflict of interest

None.

Address correspondence to: Jianping Song, The Second Affiliated Hospital of Zhejiang University School of Medicine, No. 88 Jiefang Road, Hangzhou 310000, Zhejiang, P. R. China. Tel: 86057187783-777; E-mail: zrxwk1@zju.edu.cn

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